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Cyclophosphamide and doxorubicin contribute to onset of chemobrain by altering epigenetic factors DNMT1, DNMT3A, Tet1, Mecp2, and CBP

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CYCLOPHOSPHAMIDE AND DOXYRUBICIN CONTRIBUTE TO ONSET OF CHEMOBRAIN BY ALTERING EPIGENETIC FACTORS DNMT1, DNMT3a, Tet1, Mecp2, and CBP

Master's Thesis

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Abstract

Many Breast cancer patients treated with chemotherapy have suffered from chemotherapy induced cognitive dysfunction (CICD). Studies in rats show that Erk and Akt pathways were increased upon treatment of chemotherapy. Erk and Akt are involved with normal aging of the brain and its increase hints to the source of cognitive impairment. The field of epigenetics is just beginning to surface so studying the epigenetic implications of chemobrain is reasonable. The goal of this study was to analyze common epigenetic markers and their implications in chemobrain. The prefrontal cortex (PFC) of rats with intact ovaries were used. Using RT-PCR and Western blot analysis, we have analyzed common epigenetic markers such as DNA Methyltransferase 1 (DNMT1), DNA Methyltransferase 3a (DNMT3a), Methyl-CpG binding protein 2 (Mecp2), CREB binding protein (CBP), and Ten-Eleven translocase 1 (Tet1). Differential expression of these markers between control and rats treated with chemotherapeutic drugs was observed. Chemotherapy treatment consisted of a cocktail containing doxyrubicin and cyclophosphamide, chemotherapeutics typically used for breast cancer and found to cause chemobrain. DNMT1, Tet1, CBP, and Mecp2 showed no statistically significant change in expression between saline and chemotherapy treated rat PFC. DNMT3a expression in rat PFC was found to increase with chemotherapy treatment. Global DNA methylation and hydroxymethylation assays were performed to determine the level of methylated DNA. There was no statistically significant change in DNA methylation levels and DNA hydroxymethylation levels between saline and chemotherapy treated rats. These results indicate epigenetic involvement in the development of chemobrain and is important to study further.

Introduction

Breast cancer is the most common cancer worldwide. Survival rates vary between regions of the world with a 5-year survival rate at 80% in high income countries and about 40% in low income countries.[1] There are several risk factors involved in the development of breast cancer including age, family history, and environmental factors. For example, 1:202 women from birth to age 39 will likely develop the disease compared to 1:26 women from 40-59 years. However, the detection of breast cancer has significantly improved over the years and has resulted in earlier detection of breast cancer. Imaging such as MRI, ultrasounds, and yearly mammograms are integral parts of cancer diagnosis.[2]

Thankfully, the life span of breast cancer patients has increased over the past number of years. Research is now being shifted towards long term effects of chemotherapy. Specifically, it has been previously reported that upon treatment of chemotherapeutic drugs such as doxorubicin and cyclophosphamide, patients experienced cognitive decline in the form of memory loss and learning impairment.[3] These two drugs are used commonly for breast cancer patients.[4] According the American Society of Clinical Oncology, Chemobrain effects nearly 75% of patients treated with chemotherapy for breast cancer. This loss of memory and inability to perform certain tasks that were once easy has become terrifying to patients suffering from chemobrain. Patients become less efficient at work, forget important things like looking at a stop light, or get lost while driving. Performing everyday tasks become a great burden.[5] Some doctors did address the possibility of chemobrain but never knew when or if patient's cognition would

improve. Chemobrain is a dangerous effect of chemotherapy and is fortunately becoming a major focus of study. Discovering the mechanism behind chemotherapy induced cognitive decline can help improve the quality of life for patients diagnosed with breast cancer.

This study aims to analyze common epigenetic markers to observe their role in the development of cognitive impairment following treatment of the chemotherapeutic drugs, doxorubicin and cyclophosphamide. Epigenetics literally translates as "Above" genetics in Greek referring to the layer of gene expression outside of the 4-nucleotide genetic code. Specifically, DNA is coiled around proteins called histones in part of a greater process of condensing the long DNA into a small nucleus.[6] DNA wrapped around these proteins are known as nucleosomes. Histones consist of four proteins in duplicate and are H2A, H2B, H3, H4, and one H1 protein to hold the nucleosome together. These proteins have tails attached consisting of amino acids that can be chemically modified. DNA wrapped tightly around histones are normally associated with genes that are silenced as transcription factors cannot access the DNA properly. However, DNA loosely wrapped around histones can have transcription factors bind to it activating associated genes. Histones have a positive charge because of the protein side chains allowing it to bind to negatively charged DNA. Certain modifications can take place on the histones to modify its binding on the DNA either activating genes or deactivating them.

DNA Methylation

One epigenetic mechanism takes place on the DNA itself. Specifically, cytosine can be methylated mostly when it is in a CpG site (Cytosine-Phosphodiester bond-Guanine).[7] Methylated cytosines recruit a protein known as Mecp2 which functions to silence genes.[8] This

is done in one of two ways. Mecp2 can sterically block transcription factors from binding to DNA. Mecp2 can also recruit proteins that modify and compact chromatin. As such, DNA methylation results in silencing of genes. However, new studies are revealing that Mecp2 can act as an activator of gene expression by recruiting transcription factors such as CREB.[9] The proteins responsible for this methylation are DNA Methyltransferase 1 (DNMT1) and DNA Methyltransferase 3A (DNMT3A).[7] DNMT1 is responsible for the maintenance of the methylation pattern upon DNA replication. When the DNA is replicated, the methylation pattern is not copied to the daughter strand. DNMT1 copies the methylation pattern of the parent strand to that newly formed strand. DNMT3A adds methyl groups to cytosines not previously methylated. This is important at the blastocyst stage of development after the methylation pattern of the parents have been stripped off.[10] Altered patterns of DNA methylation have been linked to the onset of cancer by silencing tumor suppressors.[11]

Until recently, methylation of cytosines was thought to be a permanent modification. (Tahiliani et.Al) found that wild type, but not mutant TET1-CD (Cytosine rich and DSBH regions) in Sf9 insect cells catalyzed conversion of 5mc to 5 hMc.[12] This modification has been shown to be linked to re-activation of the gene that was previously silenced by methylation. The proteins responsible for this conversion is the Ten-eleven-translocation enzymes (TET).[12] The hydroxymethylated cytosine is converted to 5-formylcytosine (5fC), then to carboxylcytosine (5caC) by TET1-3 enzymes. 5caC is then converted back to unmodified cytosines.[13]

Histone Acetylation

The Histones can be modified on their tail extensions protruding from the protein core.[14] Histone acetyl transferases (HATs) add an acetyl group to lysine residues on the tails of histones.[15,17] The addition of an acetyl group lessens the positive charge on the histone essentially lessening the attraction between the negatively charged DNA. This loose confirmation of the nucleosome allows DNA to be targeted by transcription factors and as a result, histone acetylation is always involved with gene activation. Histone deacetylases can reverse this process by removing the acetyl group from the histones.[16] This will result in the formation of heterochromatin and gene silencing.[15]

Histone methylation

Histone tails can also be methylated on their lysine or arginine residues in H3 or H4 proteins.[17] This modification can be involved with either gene expression or gene silencing depending on the amino acid methylated and how many methyl groups are added. Lysine or arginine residues can be methylated up to three times.[18] This modification is also reversible by histone demethylases but only on the lysine residue.[19] Methylation on the arginine residue has yet to be found as reversible.[20]

Cognition

The broad definition of cognition is the action of gaining knowledge through experiences, the senses, and thought. This includes, memory, working memory, learning, problem solving, judgement, and knowledge.[21] We have analyzed two regions of the brain responsible for certain specific behaviors and processes. Hippocampus dependent processes have already been

shown to be affected by chemotherapy by us and the lab.[3] The hippocampus is primarily responsible for the consolidation of short-term memory to long term memory, and in spatial navigation.[22] The prefrontal cortex covers the front part of the frontal lobe.[23] The prefrontal cortex is primarily responsible for executive functions such as differentiating conflicting thoughts.[24] It is also shown to play an important role in memory retrieval making it a good region to study when analyzing cognitive impairment.[25]

Chemobrain

Upon treatment of cyclophosphamide and doxorubicin, chemotherapeutic drugs used to treat breast cancer in women, patients have been affected by cognitive dysfuntion.[3] Rats treated with doxorubicin and cyclophosphamide showed significant cognitive decline when they were ovariectomized. The purpose of removing the ovaries is to eliminate the possibility of estrogen playing any type of role in cognition. This essentially mimics a post-menopausal female.[26] To test for working memory, rats treated with saline and chemotherapy were subjected to the Y-maze test.[3] Rodents tend to visit novel places and will only visit one arm of the maze once, then move to the next arm. That was seen in saline treated rats but not in chemotherapy treated rats. Treated rats showed significantly lower spontaneous alternation regardless if they were ovariectomized or intact. The study showed a decrease in working memory in rats treated with chemotherapy.[3] Spatial memory, also being hippocampus dependent, was also analyzed. There was a decreased ratio of time spent exploring the new location of the object by the time spent exploring objects on the object placement task, showing that animals treated with chemotherapy have decrease spatial memory.[3] The behavior was independent of hormonal status.[3] Upon treatment, there was significant increase in Erk 1/2 and Akt pathways normally

involved in regular aging of the brain. This study showed that these drugs can potentially advance the cellular aging process of the brain resulting from oxidative stress. Premature cellular aging has been speculated to be caused by oxidative stress that takes place upon treatment.[27] Oxidative stress increases pro inflammatory cytokines and it has been shown that epigenetic changes are associated with the increase expression of pro inflammatory cytokines.[28] Studies have shown that women with cognitive dysfunction after treatment have different leukocyte methylation patterns compared to before treatment.[29] In summary, there is reasonable evidence that treatment by chemotherapy does cause cognitive decline.

Chemotherapy

Chemotherapeutic drugs have already been shown to cause epigenetic changes. Treatment of two cancer cell lines CEM-Bcl2 and SW620 with chemotherapeutic drugs daunorubicin (DNR) and etoposide (ETOP) caused increase expression of MDR1 gene.[30] MDR1 gene is responsible for chemotherapy resistance in cancer patients.[31] Treatment of these chemotherapeutic drugs in the mentioned cancer cell lines showed decrease of MDR1 promoter DNA methylation. As methylation of DNA is associated with silencing of associated genes, decrease in methylation is associated with activation.[8] Also, there was an increase in H3K4 methylation associated with hyperacetylation at the promoter of MDR1 which indicates activation.[32] These modifications caused the abnormal increased expression of MDR1 gene in the cancer cell lines.

Another study showed that treatment of bleomycin, etoposide, and *cis*-platinum (BEP) chemotherapeutic drug used to treat testicular cancer, altered epigenetic modifications in the sperm DNA.[33] Rats treated with BEP showed a dose dependent hypermethylation of 20 loci in

germ cells. Many of these loci are hypermethylated even at low doses of chemotherapy showing that some loci are more sensitive to chemotherapy than others. Most of these loci are involved in important cell signaling pathways such as RefSeq gene Cpne8 in calcium mediated intracellular processes and RefSeq gene Slc16a2 in transport of the thyroid hormone. The increase in methylation means that these genes are abnormally repressed when the rats were treated with BEP. Also, a number of these genes are involved in tumor growth and invasion (RefSeq gene table). Alteration of the methylation patterns of these genes could affect germ cell development of the father and, as a result, have an impact on future progeny. These studies demonstrate that chemotherapy induces epigenetic modifications critical to normal biological functions.

A study evaluated the link prior chemotherapy treatment with changes in DNA methylation patterns that can explain persistent inflammation and fatigue.[34] Before radiation therapy, DNA was extracted from peripheral blood mononuclear cells (PBMCs) of 61 stage 0 IIIA breast cancer patients. These patients have received partial mastectomies with or without chemotherapy. DNA methylation was assessed at 485,000 CpG sites across the genome. Included were markers previously shown to be associated with inflammation and fatigue. Patients who received chemotherapy showed decrease methylation in 8 CpG sites compared to the non-treated counterpart. Four of these differentially methylated CpG sited are on exon 11 of transmembrane protein 49 (TMEM49), which functions to permit transport of specific molecules. This decrease in methylation of these CpG sites was associated with increase of pro inflammatory cytokine IL-6 as well as soluble tumor necrosis factor receptor. These results linked chemotherapy treatment to increase in inflammatory biomarkers. Six months post

radiation therapy, pro inflammatory markers were once again analyzed. There was still reduced methylation of 4 of the original 8 CpG sites and was associated with increase of the previously mentioned pro inflammatory cytokines. This study indicates the epigenetic changes that take place upon treatment of chemotherapy and indicates the reversible nature of some of these changes.

A recent study sought to analyze the effects of cytotoxic chemotherapeutic drugs mitomycin C (MMC) and cyclophosphamide.[35] Alterations in the transcriptome and epigenetic modifications in the mouse prefrontal cortex and the hippocampus, were assessed. Gene expression changes in response to treatment was found to be largely in females and specific to the prefrontal cortex of the brain 3 weeks after treatment.[35] Also, MMC seemed to alter gene expression more than cyclophosphamide. Treatment of MMC resulted in oxidative DNA damage as shown by the increase of 8-oxo-2'-deoxyguanosine (8-OxodG) 3 weeks after treatment. There was also a decrease in an 8-OxodG repair protein. Treatment with MMC also decreased global DNA methylation and increased global DNA hydroxymethylation in the female mouse prefrontal cortex. Most of the changes that took place in the PFC of female mice due to chemotherapy were similar to the changes that occur during normal aging. This study indicates that the effect of chemotherapy on epigenetic processes in the brain mimics aging in the brain.

Epigenetics and the brain

It has already been extensively studied that epigenetics plays a significant role in cognition. One such study showed that fear conditioning induces changes in H3K4 trimethylation (associated with activation) and H3K9 dimethylation (associated with repression) in specific

areas of the hippocampus.[36] Histone 3 methylation on Lysine 4 (associated with transcription) was analyzed 1 hour after fear conditioning. An increase in H3K4 trimethylation patterns was seen in the hippocampus compared to the control group. Fear conditioning is a test for learning and creating new memories.[37] They also analyzed the H3K9 di-methylation patterns (associated with silencing) in the hippocampus. An hour post fear conditioning, western blots revealed an increase in H3K9 di-methylation patterns. This shows that active repression is involved in memory formation. This study helps support the hypothesis that histone methylation plays a critical role in memory formation.

Another study measured mRNA levels of DNMT3A and DNMT3B.[38] Adult rats were put through fear conditioning tests. Using real-time qPCR, they looked at DNMT levels in the CA1 region of the hippocampus. DNMT3A and DNMT3B mRNA levels increased within 30 minutes of fear conditioning compared to the control group not exposed to the shock. Immediately after fear conditioning, distinct DNMT inhibitors 5-aza-deoxycytidine (5-AZA) or zebularine (zeb) were infused into the CA1 region of the hippocampus. 24 hours after infusion, memory was tested. Rats treated with DNMT inhibitors showed significantly less freezing showing that DNMT3A and DNMT3B were necessary for memory formation. However, once the inhibitor clears the hippocampus, the animals can resume normal memory formation suggesting that the change in methylation patterns is not permanent, but reversible. This study suggests that DNA methylation plays a significant role in memory formation.

Epigenetic mechanisms have been shown to modulate cognitive dysfunction in many neurodegenerative diseases such as Alzheimer's.[39] In this research, a blockade in gene expression was observed in the neurodegenerating brain. This was in large part due to the

histone deacetylase 2 which, as its name suggests, deacetylates histones and deactivates gene expression.[40] Histone deacetylase 2 was found to be significantly increased in patients with Alzheimer's disease as well as two mouse models of neurodegeneration.[40,41] The genes that are silenced as a result of histone deacetylase 2 activity are those that are involved in learning and memory. Importantly, knocking down histone deacetylase activity using short hairpin RNA will reverse the repression of these genes making them once again, active.[39] This increases synaptic plasticity and decreases memory impairment caused by neurodegeneration. This study suggests the important role of epigenetic mechanisms in normal and abnormal brain function.

Neuronal function of euchromatin histone methyltransferase (EHMT) has been evaluated in *Drosophila*.[42] EHMT is a conserved protein that methylates histone 3 lysine 9 as a repressive mark and silences transcription. Although widely expressed in the nervous system, two EHMT mutant flies are viable. These mutants are null deletions in the p element of the 5' UTR. Analysis of neurodevelopment and behavior revealed an important role of EHMT in courtship memory and on associative learning. Memory was restored when expression of EHMT was restored in adults. This indicates that the memory impairment caused by loss of EHMT is reversible. Using ChIP-seq analysis, the authors analyzed the H3K9 dimethylation profiles. There was a loss of H3K9 dimethylation in EHMT mutants in about 5% of euchromatin. This loss is also associated with genes involved with neuronal and behavioral functions that are affected in EHMT mutant flies. This study has effectively identified the role of EHMT in cognitive functions such as learning and memory. This also demonstrates the importance of epigenetics in brain functions.

Epigenetics and chemobrain

Epigenetic modifications have been shown to be a significant factor in the development of chemobrain. Rats were subjected to water maze tests in order to assess spatial learning and long-term memory of a specific location.[43] Rats were either treated with saline as a control or with a combination of three chemotherapy drugs; cyclophosphamide, methotrexate, and 5-fluorouracil. Treated rats had longer swim latencies and paths than saline rats. However, eventually the chemotherapy treated rats performed about the same as saline treated rats by day 4 of the trial.[43] The study showed that this combination of drugs leads to impairment in spatial learning and long-term memory. Using BrdU, they were also able to observe a decrease in cell proliferation in the hippocampus of rats treated with chemotherapy compared to saline treated rats. Looking at histone modification, they discovered that histone acetylation on H3 of the hippocampus and prefrontal cortex was significantly increased in rats treated with chemotherapy. Also, HDAC activity was decreased in treated rats. These are surprising results as histone acetylation is usually associated with enhanced learning and memory.[39] These results indicate that chemotherapy does induce epigenetic changes in the brain.

Furthermore, it has been shown that Chronic Kidney Disease (CKD) patients with marks of inflammation such as IL-6 (Interleukin-6) had DNA hypermethylation in peripheral blood leukocytes.[44,45] Normally, chemotherapeutic drugs cannot cross the blood brain barrier. However, studies show that pro inflammatory cytokines such as interlukin-1 (IL-1), interlukin-6 (IL-6) and tumor necrosis factor-alpha (TNFalpha) can cross the blood brain barrier.[46] Cytokine dysregulation is seen in patients experiencing chemobrain.[47] As mentioned regarding CKD inflammation, there is an increase in methylation patterns. Previous studies show that

increases in pro inflammatory cytokines has the potential to induce epigenetic changes.[28] Using this information, it therefore is reasonable to assume the increase of proinflammatory cytokines induced by chemotherapeutic drugs will cause hypermethylation of the brain as well.

Along similar lines, a study was done to determine the effects of chemotherapy exposure of a father on his transgenerational progeny's brains.[48] Male mice were treated with three chemotherapeutic drugs (cyclophosphamide (CPP), procarbazine (PCB) and mitomycin C (MCC)). DNMT1 levels and Mecp2 levels were found to be reduced in the frontal cortex of the progeny brain. Low levels of DNMT1 means lower levels of critical methylation in the transgenerational chemotherapy exposed brain.[49] Mecp2 has been shown to be required for genome stability and maintaining neuronal networks.[50] These results indicate that altered methylation in chemotherapy exposed brains can lead to cognitive dysfunction in the progeny of patients treated with chemotherapy.

These previous studies show that chemotherapy treatment post cancer results in epigenetic modifications that can cause cognitive dysfunction. The limited amount of study done in this area, as well as the surfacing knowledge of epigenetics, makes this work a reasonable focus of research.

AIM 1

In order to determine whether CICD is modulated by epigenetic mechanisms, we measured the protein expression of well characterized biomarkers; CBP, DNMT1, DNMT3a, Mecp2, and Tet1 in rats treated with chemotherapy and rats treated with saline. The model that was used in this experiment were Sprague Dawley female rats (n=26: 12 saline and 14 chemo) as the

chemotherapeutic effects on their brains has already been studied. Rats are larger in size than mice making them easier to handle. The physiology of rats is also like that of humans and has been used as models for disease.[51]

It was expected that a decrease in the DNMTs would be seen in chemotherapy treated rats against saline treated rats. This is because previous work showed that Erk and Akt pathways are activated after chemotherapy treatment so a decrease in repressing factors such as DNMTs would have been reasonable. This would align with the fact that proteins involved in aging of the brain have increased expression.[3] It was expected that CBP will have no change in chemotherapy versus saline treated rats. This is because CBP induces the expression of the neuroprotective protein Brain-derived neurotropic factor (BDNF) and BDNF levels stay the same between the two experimental groups.[3] Tet1 was expected to increase in expression in rats treated with chemotherapy as DNMTs decrease. This would align with the expected increase in gene expression of proteins involved in cellular aging of the brain. Mecp2 was expected to decrease in rat prefrontal cortex treated with chemotherapy as Mecp2 is responsible for maintaining neuronal networks.[50,55]

AIM 2

The next step was to analyze the DNA methylation patterns between the two experimental groups to determine a possible difference in methylation levels. We expected to see a decrease of global methylation in chemotherapy treated rats consistent with the predicted decrease of DNMTs. However, it would be difficult, if not impossible, to determine the genes effected by this hypomethylation.

AIM 3

As we analyzed patterns of methylation, the next step was to determine the global hydroxymethylation using a similar method as shown for Global 5-mc.

We expected to see a global increase in hydroxymethlyation in chemotherapy treated rats. This assumption was reached because as mentioned in the previous section, Tet1 conversion of 5-mC to 5hmC is associated with gene activation. As genes involved in brain aging are activated upon treatment of chemotherapy, it was expected to see an increase in activating factors such as hydroxymethylation. This would be consistent with Western blot data mentioned above. Also, as before, it is impossible to determine genes involved with this change from global hydroxymethylation data. As a result, a good next step would be to use oxidative bisulfite sequencing to determine genes linked to change in hydroxymethylation levels.

Methods

Treatment: Female rats at around 9 weeks of age were treated with a cocktail containing chemotherapeutic drugs cyclophosphamide and doxyrubicin (40 mg/kg) or with saline as a control, once per week for three weeks. Once per week injections will give the rats time to recover from the chemotherapy treatment. One week was allowed for recovery before sacrifice. Hippocampus and Prefrontal cortex were removed and frozen at -80^{o}C

Protein isolation: Extraction of Cytoplasmic and Nuclear fractions was performed (Saline; n=12 Treated; n=14 on same day) using NE-PER Nuclear and Cytoplasmic extraction reagents from Thermo Scientific. One quarter of the PFC of intact rats brain were used in the following protocol. 1% of Phosphatase inhibitors and protease inhibitors were added to the Cytoplasmic

Extraction Reagent I (CERI) and Nuclear Extraction Reagent (NER) before starting the procedure. Tissues from each sample were weighed to be 100 mg and amount of each reagent to be added were calculated. Using the manufacturer's protocol each tube received 321.54 ul CERI, 17.685 ul Cytoplasmic Extraction Reagent II (CERII), and 160.77 ul NER in total. The tissues were washed in Phosphate-Buffered Saline (PBS) and centrifuged for 5 minutes at 500 x g. Supernatant was then removed leaving the pellet as dry as possible. Tissues were homogenized in the correct amount of CERI buffer. Each tube was vortexed for 15 seconds on highest setting to suspend the pellet. They were then incubated on ice for 10 minutes. Next, ice cold CERII was added to each sample tube, vortexed for 5 seconds on highest setting, and incubated on ice for 1 minute. Sample tubes were vortexed again for 5 seconds on the highest setting and centrifuged for 5 minutes at maximum speed. Supernatant containing cytoplasmic extract was removed and stored in -80 C˚. The pellet was suspended in NER buffer and vortexed for 15 seconds. The samples were placed on ice and vortexing continued every 10 minutes for 15 seconds for a total of 40 minutes. Samples were centrifuged at maximum speed for 10 minutes and the supernatant was transferred to clean tubes. Nuclear fractions were stored in -80 C˚

Protein quantification: Pierce™ BCA Protein Assay Kit was used to quantify protein concentration of all samples (done on same day). Protein reduce Cu^{2+} to Cu^{1+} in alkaline medium. Using a unique reagent containing bicinchoninic acid, a purple color change will take place because of chelation of two BCA molecules to one cuprous ion. The intensity of the color change is proportional to the protein concentration in the sample. The 96 well plate is put into a spectrophotometer to quantify the protein concentration based on color Standards were prepared as per Table 1.

Dilution Scheme for Standard Test Tube Protocol and Microplate Procedure (Working Range = 20-2,000µg/mL)

Vial	Volume of Diluent (µL)	Volume and Source of BSA (µL)	Final BSA Concentration (µg/mL)
A	0	300 of Stock	2000
B	125	375 of Stock	1500
C	325	325 of Stock	1000
D	175	175 of vial B dilution	750
E	325	325 of vial C dilution	500
F	325	325 of vial E dilution	250
G	325	325 of vial F dilution	125
H	400	100 of vial G dilution	25
I	400	0	0 = Blank

Table 1: Preparation of the standard curve

The working reagents were prepared by adding 50 parts of BCA reagent A to 1-part BCA reagent B. 25 microliters of each standard or sample were added into a microplate well. Unknowns were done in duplicates. 20 ul of the working reagent were added into each loaded well and mixed for 30 seconds. Plate was covered with parafilm and incubated at 37 C˚ for 30 minutes. Absorbance was read at 562 nm on the Specramax M5 plate reader. Adjusted concentration was determined by the machine.

Western Blot: Before beginning the western blot procedure, calculations were performed to ensure the same amount of protein (30 mg) from each sample would be added in to each well. This was done in the following way; 20ul of total solution would be added to each tube. 5.1 ul of sample buffer (Alfa Aesar Laemmli Sodium Dodecyl Sulfate (SDS) sample buffer 4X), Lysis buffer (1X RIPA), and lysate. The number of micrograms of protein to be used divided by the adjusted concentration multiplied by 1000. This gives the amount of lysate needed. Multiply that by 1.02 to account for pipetting errors. 15 minus the amount of lysate gave the amount of lysis buffer to add. That number was also multiplied by 1.02 for pipetting error. The total volume was slightly above 20 ul. The solution was then heated to 98 C˚ for 5 minutes using the Techne TC-312

thermocycler. On any given day, six of the saline samples and seven of the chemotherapy treated samples were added to one gel to ensure daily consistency.

Nuclear fractions were prepared as described above. Thermo Broad range or High range spectra ladder were run alongside the prepared samples in an acrylamide gel all in running buffer. Running buffer contained 100 ml of 10x Tris-Glycine, 20 ml 10% SDS, and 890 ml dH_2O. The gel ran at 150 volts for 1 hour or until the blue loading line was just at the bottom of the gel. The separated proteins were transferred to a PVDF membrane for 90 minutes at 100 volts. Transfer buffer consisted of 100 ml 10x Tris-Glycine, 200 ml methanol and 700 ml dH_2O. The membrane was activated in methanol for 1 minute before the transfer was set up. Membranes were blocked in 5% Bovine Serum Albumin (BSA) for 1 hour at room temperature to reduce non-specific binding. Primary antibody incubation was done overnight in the cold room of 4 C˚ using 5% BSA, 0.1% tween 20, and one of the following; 1:500 rabbit anti-DNMT1, 1:500 rabbit anti-DNMT3a, 1:1000 rabbit anti-Lamin b, and 1:1000 rabbit anti-Mecp2. Membranes were washed 4 times for five minutes each with wash buffer (999 ml 1x TBS and 1 ul of tween 20). Secondary antibody incubation was done for 1 hour at room temperature and contained 5% BSA, 0.1% tween 20, and 1:3000 anti-rabbit HRP (horseradish peroxidase)-conjugated antibody. 4 washes with wash buffer for five minutes followed the secondary antibody incubation. Chemiluminescent substrate from Pierce was added to the membranes (Protein side) and allowed to incubate for 5 minutes at room temperature. The fluorescent signal was detected using Thermo Scientific CL-Xposure film and the resulting bands were analyzed using imageJ software.

DNA Isolation: QIAamp® Fast DNA Tissue Kit was used to isolate the DNA fraction from the brain tissue. Tissues from each sample was cut and weighed to be between 5-25 mg and was placed in

disruption tubes. As per manufacture's protocol, 200ul of AVE buffer, 40 ul of VXL buffer, 1 ul of DX reagent, 20 ul of proteinase K, 4 ul RNase A were all added to each tube. The tubes were vortexed at full speed for 5 minutes to homogenize the tissues. The samples were placed in the New Brunswick Scientific incubator shaker at 500 RPM for 20 minutes at 56 C˚ 265 ul Buffer MVL was added to each sample and mixed by vortexing. The mixture was added to the spin columns provided and centrifuged for 1 minute at full speed. The columns were then placed in clean collection tubes and the tubes containing the filtrate were discarded. 500 ul of buffer AW1 was added to each sample spin column and were centrifuged for 30 seconds at full speed. The collection tubes were discarded and 500 ul of buffer AW2 was added to the spin columns. The columns were centrifuged for 30 seconds at full speed and the collection tubes were discarded. New collection tubes were added, and the tubes were centrifuged for 2 minutes. The collection tubes were discarded and replaced by microcentrifuge tubes. 100 ul of ATE were added to the spin columns, incubated at room temperature for 1 minute, and centrifuged for 1 minute at full speed. The DNA concentration was then analyzed using the Thermo Scientific Nanodrop 2000.

Global DNA Methylation: MethylFlash™ Global DNA Methylation (5-mC) ELISA Easy Kit (Colorimetric) from Epigentek was used to measure global 5-mC. First, 1 ul of PC solution was diluted in 9 ul of Negative Control (NC) solution to make diluted PC. Standard curve is prepared by adding a specific amount of Positive Control (PC), diluted PC, and NC to make the following percent of PC/well; 0.1%, 0.2%, 0.5%, 1.0%, 2.0%, and 5.0%. For the negative control, 100 ul of Binding Solution (BS) buffer was added to the strip wells and 2 ul of NC. For positive control, 100 ul BS buffer was added into its respective wells and 2 ul of PC at different concentrations (0.1%-5%) to generate the standard curve. For the samples (All 26 samples were used on the same

plate), 100 ul of BS buffer was added along with 100 ng of sample DNA. The plate was tapped gently to ensure the bottom of the substrate containing well was coated with solution. It was covered in parafilm and incubated at 37 C˚ for 60 minutes. Each well was then washed with diluted wash buffer three times. The remaining wash buffer was emptied and 50 ul of the 5-mC Detection Complex Solution (Containing 1 ml diluted wash buffer, 1 ul mcAB, 1 ul of SI buffer, and 0.5 ul of Enhancing Solution (ES) buffer) was added to each well. The wells were incubated at room temperature for 50 minutes. The 5-mC detection solution was emptied and the wells were washed five times with the wash buffer. 100 ul of Developer Solution (DS) solution was added to each well in a vertical fashion using a multipipette and color changes were observed. After 10 minutes, 100 ul of Stop Solution (SS) buffer was added to each well in the manner and the plate was read in the Specramax M5 plate reader at 450 nm. Calculations were performed to get the percentage 5-mC.

Global DNA Hydroxymethylation: MethylFlash™ Global DNA Hydroxymethylation (5-hmC) ELISA Easy Kit (Colorimetric) was used to measure global 5-hmC. First, 1 ul of PC solution was diluted in 9 ul of NC solution to make diluted PC. Standard curve is prepared by adding a specific amount of PC, diluted PC, and NC to make the following percent of PC/well; 0.01%, 0.04%, 0.10%, 0.20%, 0.40%, and 1.0%. For the negative control, 100 ul of BS buffer was added to the strip wells and 2 ul of NC. For positive control, 100 ul BS buffer was added into its respective wells and 2 ul of PC at different concentrations (0.02%-1%) to generate the standard curve. For the samples (All 26 samples were used on the same plate), 100 ul of BS buffer was added along with 100 ng of sample DNA. The plate was tapped gently to ensure the bottom of the substrate containing well was coated with solution. It was covered in parafilm and incubated at 37 C˚ for 60 minutes. Each

well was then washed with diluted wash buffer three times. The remaining wash buffer was emptied and 50 ul of the 5-hmC Detection Complex Solution (Containing 1 ml diluted wash buffer, 1 ul hmcAB, 1 ul of SI buffer, and 0.5 ul of ES buffer) was added to each well. The wells were incubated at room temperature for 50 minutes. The 5-hmC detection solution was emptied and the wells were washed five times with the wash buffer. 100 ul of DS solution was added to each well in a vertical fashion using a multipipette and color changes were observed. After 10 minutes, 100 ul of SS buffer was added to each well in the manner and the plate was read in the Specramax M5 plate reader at 450 nm. Calculations were performed to get the percentage 5-hmC.

RNA Isolation: RNA isolation was done using RNeasy Mini Kit from Qiagen. 30 mg of rat PRC samples were weighed and homogenized in 60 ul of RLT buffer. 600 ul of 70% ethanol was added and mixed well by pipetting up and down. 700 ul of the samples were transferred to included spin columns and centrifuged at 8000 x g for 15 seconds. The flow through was then discarded and new collection tubes were added. 700 ul of RW1 buffer was added to each spin column and centrifuged for 15 seconds at 8000 x g. The flow through was discarded. 500 ul buffer RPE was added to each spin column and centrifuged for 15 seconds at 8000 x g. Flow through was discarded. 500 ul buffer RPE was added to each spin column and centrifuged for 2 minutes at 8000 x g. Flow through was discarded and 1.5 ml collection tubes were placed on the spin columns. 50 ul of RNase-free water was added to each spin column and centrifuged for 1 minute at 8000 x g to elute the RNA. RNA concentration was determined using the Thermo Scientific Nanodrop 2000. RNA was stored in -80˚C until use.

cDNA Synthesis: The Thermo Scientific Verso cDNA Synthesis Kit was used to reverse transcribe 2 ug of RNA to cDNA. 2 ug of RNA (six saline and six chemotherapy treated) was prepared by

diluting the RNA in a specific amount of Diethyl Pyrocarbonate (DEPC) treated water such that 2 ul of RNA would be 2 ug. 2 ul of RNA of each sample were first heated to 65 C˚ for 5 minutes in a thermocycler. A master mix was prepared in which each 2 ul of diluted RNA would receive 8 ul of 5X RT buffer, 1.33 ul Random hexamers, 0.67 ul Anchored oligo dT primers, 4 ul dNTPs, 2 ul RT enhancer, and 2 ul of Verso enzyme. The master mix was then added to the post-heated RNA. The resulting mixture underwent cDNA synthesis in a thermocycler at 42 C˚ for 45 minutes, then 2 minutes of inactivation at 95 C˚. The cDNA was stored in -80˚C until use.

RT-PCR: PowerUp SYBR Green Master Mix from applied biosystems was used for RT-PCR. A master mix was made so that each well received 1X SYBR Green master mix solution, 1 ul of 10 um forward primer, 1 ul of 10 um reverse primer, and 6 ul of DEPC treated water. However, for 18S, only 0.5 ul of each primer was added and 7 ul of DEPC treated water. Primers used were from Invitrogen and are listed in (table 2). 18 ul of the master mix was added to each wells on a 96 well PCR reaction plate along with the 2 ul of each cDNA sample (six saline and six chemotherapy treated). cDNA was not placed in wells assigned for negative control. Samples were added in duplicate and ThermoScientific optical adhesive film was added to the top of the plate. The plate was placed in the Biosystems 75000 real-time PCR software system after set-up. The cycling settings were set to 95 C˚ melting temperature for 15 seconds, annealing temperature 3-5 C˚ below the melting temperature of primers for 1 minute. It was then heated to 72 C˚ for 1 minute before heated up to the melting temperature. The total amount of cycles was 40. Amplification was monitored as comparative Ct values. Data was analyzed and presented as relative fold change.

Target	Primer sequence 5'→3'
Tet1	(+)GAGCCTGTTCCTCGATGTGG (-)CAAACCCACCTGAGGCTGTT
CBP	(+)AAGCAACAAATTGGGTCACCAGGC (-)TACTAAGGGATGTGGCGATCTGCT
18S	(+)CATTCGAACGTCTGCCCTAT (-)GTTTCTCAGGCTCCCTCTCC

Table 2: List of primers used for RT-PCR

Results

Chemotherapy induced changes in expression of different epigenetic markers

To test the effect of chemotherapy on epigenetic modifiers, expression of DNMT3a, DNMT1, and Mecp2 were determined by western blot analysis. The bands were quantified and arranged graphically. There was no statistically significant change in DNMT1 expression upon treatment of chemotherapy (Figure 1a). However, there was a statistically significant increase in DNMT3a (140%) expression upon chemotherapy treatment (Figure 1b). Mecp2 expression showed no statistically significant change in Mecp2 expression upon chemotherapy treatment (Figure 1c). CBP and Tet1 were assessed using RT-PCR. The RT-PCR data showed that the expression of CBP and Tet1 remained unchanged rats treated with chemotherapy compared to controls. (Figure 2). Results from Tet1 and CBP were not found to be statistically significant.

Chemotherapy induces changes in global DNA methylation and hydroxymethylation

Global DNA methylation and hydroxymethylation levels were also analyzed. There was no statistically significant change in 5-mC and 5-hmC levels in the chemotherapy treated rats compared to controls (Figure 3a and 3b).

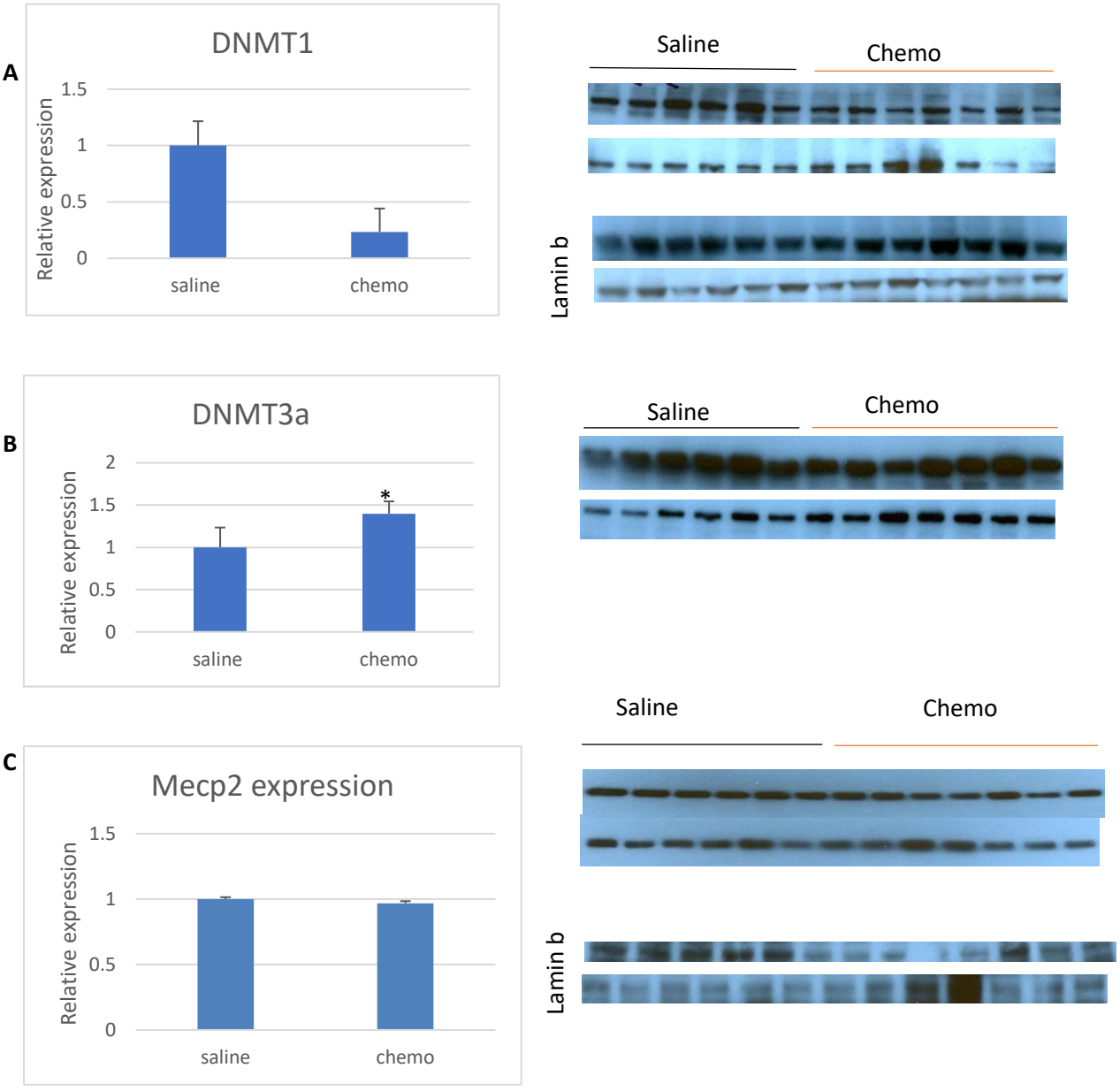


Figure 1: *Western Blot analyses of protein expression in PFC of chemo and saline treated rats.* Graphically shown on the left and the bands are shown on the right. Error bars represent standard error. Saline (n=12) and chemo (n=14). Unpaired t-test; DNMT1 p=0.2433 (NS), DNMT3a p=0.0005, Mecp2 p=0.4577 (NS)

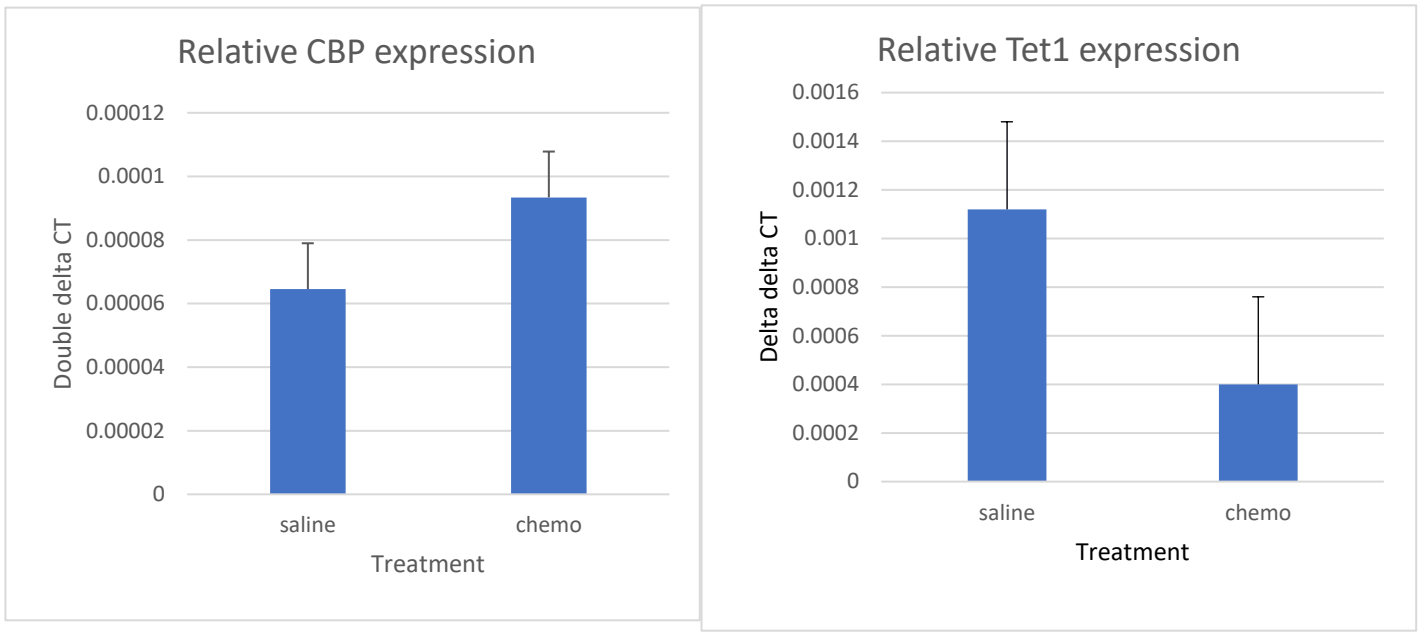


Figure 2: *RT-PCR analysis of CBP and Tet1 in the PFC of treated and control rats.* Error bars indicate standard error. Chemo n=12, Saline n=12. Unpaired t-test; CBP p=0.3648 (NS), Tet1 p=0.4981 (NS)

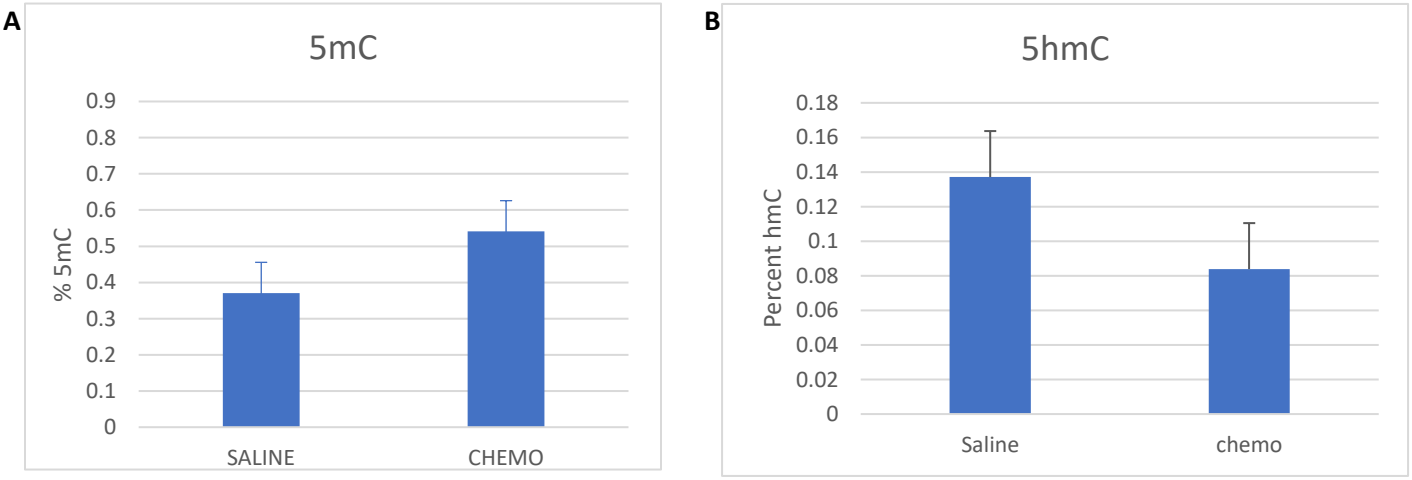


Figure 3: *Global DNA methylation and hydroxymethylation in PFC of saline and chemo treated rats.*. Error bars signify standard error. Saline n=12, Chemo n=14. Unpaired t-test; 5-mC p=0.0921, 5-hmC p=0.5656

Discussion

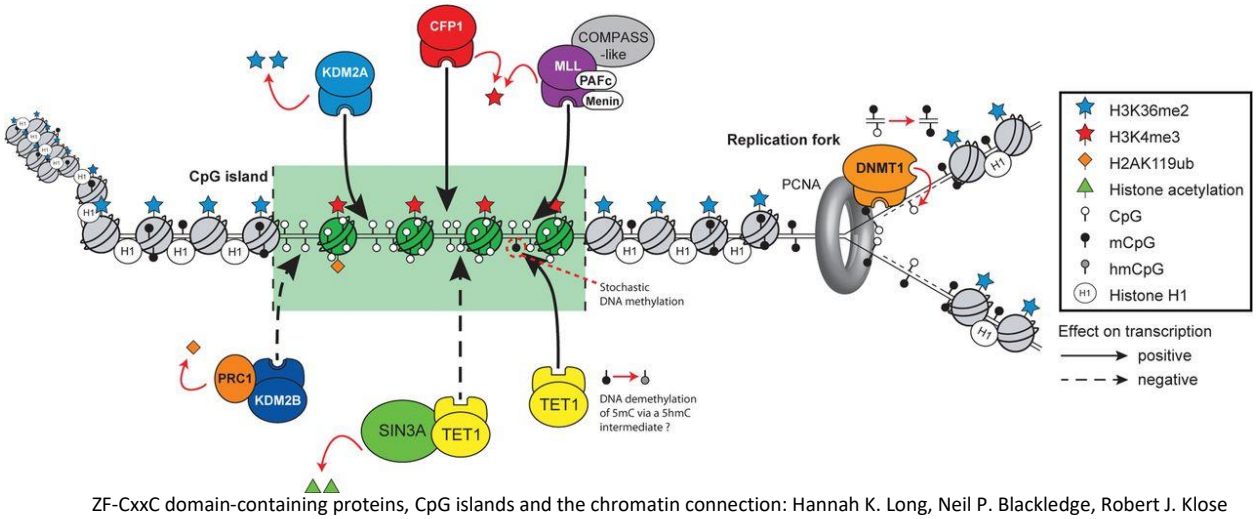
Specific chemotherapy treatments have been shown to cause cognitive decline in breast cancer patients. This study analyzed epigenetic implications in the development of chemotherapy induced cognitive decline post doxorubicin and cyclophosphamide treatment. Epigenetic markers include; DNMT1, DNMT3a, Mecp2, CBP, and Tet1.

DNMT1

DNMT1 is a DNA methyl transferase responsible for methylating cytosines on DNA in a manner that maintains the pattern of the parent strand during replication (Figure 4).[7] This will ensure proper methylation throughout further generations of the DNA. Methylation of cytosines has a repressive effect on the genes associated with it.[8] Mecp2 will be recruited to the methylated cytosine which will inhibit transcription of the gene either by sterically not allowing transcription factors to bind, or by recruiting proteins that modify and compact chromatin.[8] As such, this is a protein that is important to study as genes such as Erk and Akt are misregulated after treatment with chemotherapy.[3] The results showed no statistically significant change in DNMT1 protein expression in chemotherapy versus control samples. A decrease in DNMT1 expression was expected as less DNMT1 would perhaps indicate less methylation, fitting with the known increase of ERK and AKT.

Previous studies have analyzed the effects of chemotherapy on DNMT1.[53] Bcl-2/E1B 19-kDa interacting protein 3 (BNIP3) is a proapoptotic protein with often low expression in colorectal

cancer.[53] This is because of increased methylation of the gene promoter. It is known that the combination of chemotherapy and radiation induce apoptosis in colorectal cancer cells, but the molecular mechanism is unknown.[54] Treatment of colon cancer cells with chemotherapy (5-fluorouracil, oxaliplatin, and irinotecan) and radiation increased expression of proapoptotic protein BNIP3.[53] The chemotherapy treatment also decreased the expression of DNMT1 even without the radiation therapy. This study can indicate that chemotherapy decreases the expression of DNMT1 and could explain the western blot trend found in the results above.



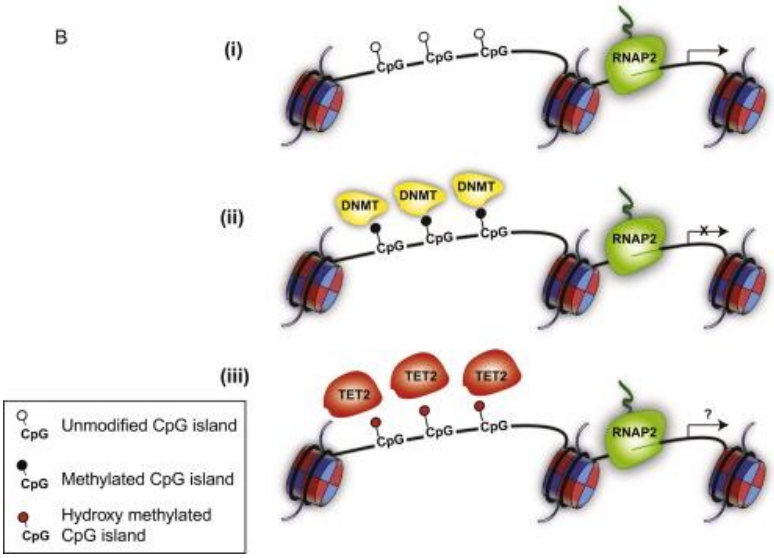
ZF-CxxC domain-containing proteins, CpG islands and the chromatin connection: Hannah K. Long, Neil P. Blackledge, Robert J. Klose

Figure 4 shows the activity of DNMT1. DNMT1 acts at the replication fork and ensures that the correct pattern of DNA methylation is carried over to the next generation strand

DNMT3a

DNMT3a is a protein involved with de novo methylation (Figure 5).[7] This means that cytosines can be methylated by these proteins independent of their parent strand of DNA. Our results showed a slight but statistically significant increase (140%) of DNMT3a protein expression in chemotherapy treated rat brains versus control rat brains. This result is interesting as data from Fathema Uddin, a former student in the lab, showed a decrease of DNMT3a protein expression

in ovariectomized rats treated with chemotherapy compared to ovariectomized controls (Uddin and Hubbard, unpublished data). The difference may be explained by the presence of estrogen. Nevertheless, this global increase of DNMT3a expression perhaps suggests the inhibition of specific genes that are yet to be determined.



The Molecular Basis of Cancer (Fourth Edition), 2015

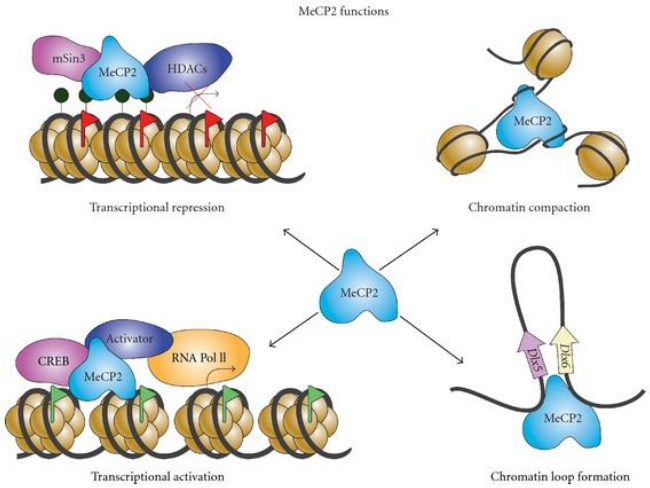
Figure 5 shows the activity of DNMT3a. DNMT3a methylates cytosines in the CpG islands in the promoter of genes. This results in silencing of the associated gene. Also shown is the activity of Tet1 enzyme that converts methylated cytosine to hydroxymethylated cytosine to reactivate the gene.

Mecp2

Mecp2 as mentioned earlier, is a protein that is responsible for the silencing of genes (Figure 6).[8] Mecp2 will bind to methylated cytosines and inhibit transcription by either sterically not allowing transcription factors to bind, or by recruiting proteins that modify and compact

chromatin.[8] Our data suggested no statistically significant change in Mecp2 expression in chemotherapy treated rat PFC versus controls.

Kovalchuk A et. Al (2016) showed that treatment of chemotherapeutic drugs (cyclophosphamide, procarbazine, and mytomycin c) results in a decrease expression of Mecp2 in the subject progeny.[48] Mecp2 is essential for maintaining neuronal networks and brain anatomy.[55] Neurological diseases such as Rett syndrome involve a mutation of Mecp2.[56] It might be reasonable to assume that rats treated with chemotherapy resulting in chemobrain, would have a similar decrease in Mecp2. However, figure 6 above showed otherwise. Kovalchuk A et. Al (2016) used treated male mice then mated with untreated females and the brains of the one-week old progeny were then analyzed. However, female intact rats were used in this experiment. Perhaps the presence of estrogen had a different effect on Mecp2. The effects on Mecp2 need to be further studied.



Linking Epigenetics to Human Disease and Rett Syndrome: The Emerging Novel and Challenging Concepts in MeCP2 Research Fig 1

Figure 6 Shows the activity of Mecp2 and how it silences genes by chromatin compaction

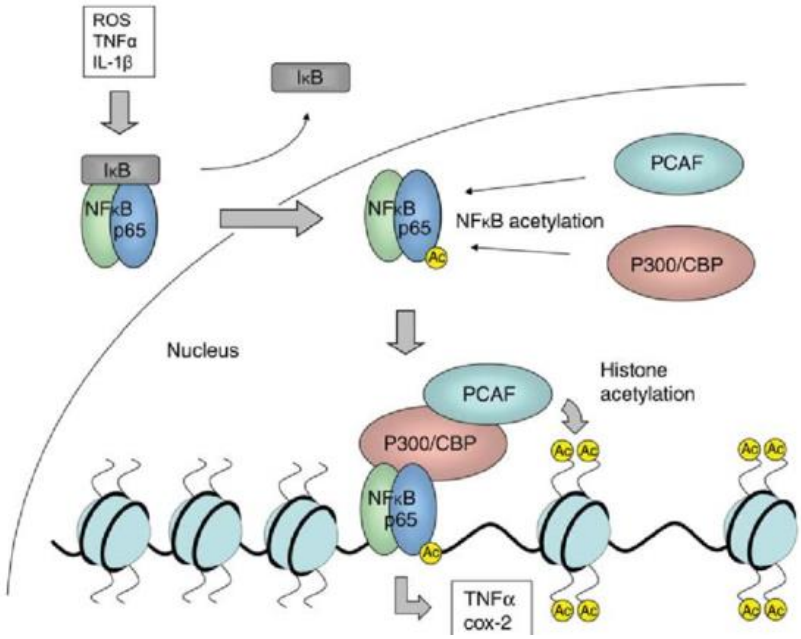
CBP

CREB binding protein is a coactivator that positions histone acetyltransferases near associated nucleosomes.[57] As such, CBP is involved with the activation of genes.[15] One of these genes known to be activated by CBP is BDNF, a protein involved in growth and maintenance of neurons.[58] We have previously shown that the expression level of BDNF does not change between control and treated of intact rats or ovariectomized rats.[3] However, between intact and ovariectomized, BDNF levels increase significantly.[3] This change can be caused by the stress of removing the ovaries. As BDNF levels do not change between saline and chemotherapy treated rats, it would be expected that CBP expression levels do not change either. In figure 2, we observed no statistically significant change of CBP. This trend perhaps ties in with previous BDNF data but not with other CBP target genes. One such gene is the pro inflammatory cytokine TNF-Alpha.[59] Perhaps an increase of CBP in chemotherapy treated rat's PFC would lead to an increase in TNF-Alpha and, as mentioned earlier, the increase of pro inflammatory cytokines can have effects on epigenetics. As mentioned earlier, peripheral blood leukocytes with increase pro inflammatory cytokines had global DNA hypermethylation.[44,45]

This data from CBP is also not in line with previous data. Results by Fathema Uddin (unpublished data) showed that there was a significant decrease in CBP between control and chemotherapy treated in the prefrontal cortex of ovariectomized rats. This makes sense as CBP mediates adult neurogenesis, a decrease of CBP when treated with chemotherapy would indicate decrease neurogenesis resulting in cognitive decline. The data observed here showed

no statistically significant change in CBP upon chemotherapy treatment. This may be due to the presence of estrogen in intact animals.

As previously mentioned, Creb binding protein acts as a histone acetyltransferase[57] and is associated with the activation of genes[15]. We therefore attempted to analyze the histone acetyltransferase activity in chemotherapy treated rat PFC and controls. However, the data was inconclusive possibly due to the use of frozen samples as opposed to fresh samples.



Epigenetic histone acetylation modifiers in vascular remodelling: New targets for therapy in cardiovascular disease fig 2

Figure 7 shows the activity of CBP. CBP acts as a coactivator and has histone acetyltransferase activity. This function allows it to activate target genes such as inflammatory cytokine TNF-Alpha and BDNF.

Tet1

As previously mentioned, Tet1 is involved with the reactivation of genes previously silenced by DNA methylation (Figure 5).[12] In the qRT-PCR results, no statistically significant change in Tet1 RNA expression was observed in the PFC between saline and chemotherapy treated rats. This is not in line with expectations as an increase in Tet1 would lead to an increase in 5-hmC activating factors, in line with known increased ERK and AKT. Oxidative bisulfite sequencing would give us a better understanding as to which genes are affected by a possible change on 5-hmC levels not detected in this study.

However, recent studies have found negative correlations between Tet1 expression and 5hmC levels.[61] When studying Tet1 and Tet2 expression in aging mice hippocampus, it was found that there was an increase in 5hmC but decrease in Tet1. As TET1 levels increase, 5hmC levels decrease. The negative correlation can be because Tet1 converts 5mC to hmC which is in turn further converted to 5CaC.[12]

Global DNA methylation/hydroxymethylation

The results for Global DNA methylation showed no observed statistically significant change in DNA methylation in chemotherapy treated rats (Figure 3). This is perhaps not in line with DNMT3a expression increase in chemotherapy treated rats. However, there are other DNMTs that function to methylate DNA besides DNMT3a, the presence of which can affect 5-mC levels. Which genes were affected are unknown, however, further work such as oxidative bisulfite sequencing would determine the genes being affected by a possible change in methylation not observed in this study.[62]

A recent study showed that DNMT3a and TET1 compete to regulate the epigenetic landscape of mouse embryonic stem cells.[63] DNMT3a seems to be enriched at distant promoters but not proximal promoters where Tet1 exhibits binding. Deletion of Tet1 generated an increase in DNMT3a1 binding at genes where wild type Tet1 was found. However, deletion of DNMT3a had an insignificant effect on Tet1 binding showing that Tet1 can protect DNA from methylation by preventing DNMT3a from binding.

Global DNA hydroxymethylation results indicated no observed statistically significant change in DNA hydroxymethylation in chemotherapy treated rat PFC. Hydroxymethylation as mentioned earlier, is an oxidation step that takes place on methylated cytosine to return the modified cytosine to its original unmodified state.[12] This will reactivate transcription and the gene associated with it.[12] No observed change in hydroxymethylation could perhaps be in line with the Tet1 data. Once again, which genes were affected by a possible change in DNA hydroxymethylation is unknown as this is a global DNA analysis. Further work such as oxidative bisulfite sequencing will determine the genes being affected by a possible change in 5-hmC not observed in this study.

Future Direction

It would be a good next step to look at the activity of HDAC in the chemobrain and perhaps inhibiting it. Previous studies do indicate that inhibiting HDAC can have the same results as enhancement environments in mice that underwent neurodegeneration.[52] In said study, treatment of HDAC inhibitors increases histone acetylation, leads to recovery of long-term memories, and reinstates the learning ability of mice. It would therefore be interesting to see if

treatment of HDAC inhibitors to patients suffering from chemobrain would reverse the effects and restore normal cognitive function.

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